

# Activation of the MKK4-JNK pathway during erythroid differentiation of K562 cells is inhibited by the heat shock factor 2- $\beta$ isoform

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**Abstract** In this study we report the activation of c-Jun N-terminal kinase (JNK) in human K562 erythroleukemia cells undergoing hemin-mediated erythroid differentiation, which occurs concomitantly with activation of heat shock factor 2 (HSF2) and leads to a simultaneous *in vivo* phosphorylation of c-Jun. The activation of JNK occurs through activation of mitogen-activated protein kinase kinase (MKK) 4 and not by activation of MKK7 or inhibition of JNK-directed phosphatases. We have previously shown that overexpression of the HSF2- $\beta$  isoform inhibits the activation of HSF2 upon hemin-induced erythroid differentiation. Here we demonstrate that HSF2- $\beta$  overexpression blocks the hemin-induced activation of the MKK4-JNK pathway, suggesting an erythroid lineage-specific JNK activation likely to be regulated by HSF2. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Heat shock factor 2; JNK/SAPK; Erythroid differentiation; Hemin; K562 cell; Mitogen-activated protein kinase

## 1. Introduction

The JNK/SAPK pathway is a member of the mitogen-activated protein kinase (MAPK) pathways and is known to be activated by pro-inflammatory cytokines and by physical and chemical stresses, as well as by apoptotic signals [1]. Three genes, c-Jun N-terminal kinases (JNKs) 1, 2 and 3, encoding 10 JNK isoforms have been cloned. Of these, JNKs 1 and 2 are abundantly expressed, whereas JNK3 is more highly expressed in neural tissues [2]. Activation of JNKs can occur by activation of the MAPK kinases MKK4/SEK1 or MKK7 [1], or alternatively by inhibition of JNK phosphatases [3]. Although more information on the role of JNK in apoptosis and embryonic morphogenesis is accumulating, the role of JNK signaling during differentiation-related processes has remained elusive [4].

K562 human erythroleukemia cells have been widely used as a cellular model for hematopoietic differentiation, as they

can be stimulated to differentiate along megakaryocytic, erythroid, and to a lesser extent, monocytic lineages [5,6]. In response to hemin, K562 cells start to differentiate along the erythroid lineage, as characterized by elevated expression of embryonic and fetal globins [7]. While MAPK/ERK signaling has been shown to be important for the megakaryocytic differentiation [8], relatively little is known about signaling events during erythroid differentiation.

HSF2 is a member of the heat shock transcription factor family known to regulate transcription of genes encoding heat shock proteins (Hsps) [9]. In contrast to the classical stress-responsive heat shock factor, HSF1, abundant expression and activation of HSF2 have been associated with development- and differentiation-related processes [10–16]. Curiously, no spatial or temporal correlation between activation of HSF2 and expression of Hsps has been shown during mouse embryogenesis, heart development or rat spermatogenesis [13,14,16], which has raised the possibility that HSF2 may have target genes distinct from those of HSF1 [17,18]. HSF2 exists as two alternatively spliced isoforms HSF2- $\alpha$  and HSF2- $\beta$ , displaying tissue-specific expression and distinct transcriptional activities [19]. We have earlier demonstrated that HSF2 expression and acquisition of HSF2 DNA binding activity are strictly specific for the erythroid characteristics of K562 cells, since HSF2 is rapidly downregulated during megakaryocytic differentiation [15]. This indicates that HSF2 could have a role as a lineage-specific regulator of erythroid differentiation. Here we show that JNK is strongly activated concomitantly with the activation of HSF2 during hemin-induced erythroid differentiation of K562 cells. The JNK activation is exclusively mediated by MKK4 and not by MKK7 or phosphatase inhibition, and it leads to a simultaneous *in vivo* phosphorylation of c-Jun. Interestingly, overexpression of the HSF2- $\beta$  isoform, which has been previously shown to abolish the erythroid lineage-specific activation of HSF2 and suppress the erythroid differentiation [20], also prevents the observed activation of JNK, suggesting a novel, HSF2-mediated mechanism of JNK regulation during erythroid differentiation of K562 cells.

## 2. Materials and methods

### 2.1. Cell culture

Human K562 erythroleukemia cells were cultured in a humidified

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5% CO<sub>2</sub> atmosphere at 37°C in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin). K562 cells stably overexpressing HSF2- $\alpha$  and HSF2- $\beta$  isoforms (2 $\alpha$ -C7 and 2 $\beta$ -D5, respectively) [19] were maintained in RPMI 1640 containing G418 (500  $\mu$ g/ml; Gibco BRL). For experimental treatments, the HSF2- $\alpha$ - and HSF2- $\beta$ -overexpressing cells were plated in RPMI 1640 without G418. Hemin (Aldrich) was added to a final concentration of 40  $\mu$ M, anisomycin (Calbiochem) to 5.0  $\mu$ g/ml, and TPA (Sigma) to 10 nM. For osmotic shock treatments, cells were incubated for 10 min in medium containing 0.5 M NaCl. Heat shock was performed at 42°C in a water bath. For UV irradiation, cells were exposed to a 15 W UV light for 5 min.

## 2.2. Plasmid constructs and transfection

The plasmid for the bacterial expression of JNK1 was a gift from Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA, USA) and the plasmid for expression of GST-c-Jun was a gift from Dr. James Woodgett (Ontario Cancer Institute, Toronto, ON, Canada). For measurement of kinase activities, cells were transfected with wild-type GST-tagged MKK4/SEK1 (pEBG-SEK1) [21] or with wild-type FLAG-tagged MKK7 (a kind gift from Dr. James Woodgett). HSF2- $\alpha$ -Myc plasmid is described in detail elsewhere (Alastalo et al., in preparation). For transfections  $5 \times 10^6$  cells were suspended in 0.4 ml OptiMEM (Gibco BRL) and 10–50  $\mu$ g DNA was added. The cells were then subjected to a single electric pulse (975  $\mu$ F, 200 V) in 0.4 cm gap electroporation cuvettes (BTX) followed by dilution to  $5 \times 10^5$  cells/ml in RPMI 1640 containing 10% FCS and antibiotics.

## 2.3. SDS-PAGE and Western blotting

$3 \times 10^6$  cells were lysed in Laemmli sample buffer and sonicated to reduce sample viscosity. 5  $\mu$ l of samples were separated on a 10% SDS polyacrylamide gel and transferred to nitrocellulose membrane (Protran nitrocellulose; Schleicher and Schuell) using a semi-dry transfer apparatus (Bio-Rad). Western blotting was performed using antibodies against phospho-JNK (Promega), c-Jun (New England Biolabs), JNK1(FL) (Santa Cruz), HSC70 (StressGen), and HSF2 (a kind gift from Dr. Richard I. Morimoto, Northwestern University, Evanston, IL, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega and Zymed. The blots were developed using the enhanced chemiluminescence method (ECL; Amersham).

## 2.4. Gel mobility shift assay

A gel mobility shift analysis of protein–DNA complexes was performed as described previously [22]. Briefly, whole cell extracts (12  $\mu$ g protein) were incubated with a <sup>32</sup>P-labeled oligonucleotide representing the proximal HSE of the human hsp70 promoter. The protein–DNA complexes were resolved on a 4% non-denaturing polyacrylamide gel.

## 2.5. In vitro immunocomplex kinase assay for JNK

Cells for JNK assays were lysed in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM dithiothreitol (DTT) in phosphate-buffered saline, pH 7.4). Transfected cells were harvested 48 h post-transfection. Insoluble material was removed by centrifugation, and the cell lysate was incubated with an antibody specific for JNKs (Santa Cruz), together with protein A Sepharose (Sigma) at 4°C overnight. The beads were washed three times with the lysis buffer, three times with LiCl buffer (500 mM LiCl, 100 mM Tris, pH 7.6, 0.1% Triton X-100, 1 mM DTT in H<sub>2</sub>O), and three times with JNK kinase assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 1 mM DTT in H<sub>2</sub>O). Immuno-precipitates were mixed with 5  $\mu$ g of GST-c-Jun, 25  $\mu$ mol ATP, 50 mM MgCl<sub>2</sub> and 2.5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP in 40  $\mu$ l kinase buffer followed by a 30 min incubation at 37°C. The reactions were terminated by boiling in Laemmli sample buffer. Samples were resolved on 10% SDS-PAGE and autoradiographed.

## 2.6. Two-step immunocomplex kinase assay for MKK4 and MKK7

K562 cells were transfected with tagged wild-type MKK4 or MKK7 kinase constructs and the assay was done 40 h after the transfection to measure kinase activity. The cells were lysed in lysis buffer (20 mM Tris pH 7.4, 10% glycerol, 1% Triton X-100, 137 mM NaCl, 25 mM  $\beta$ -glycerophosphate, 2 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF). Cleared cell lysate was incubated overnight with 40  $\mu$ l of GSH-Sepharose beads (Sigma) for MKK4 or 2.5  $\mu$ g M2 anti-FLAG antibody (Sigma) and 40  $\mu$ l of protein G Sepharose beads (Amersham) for MKK7. The beads were washed three times with lysis buffer, three times with LiCl buffer, and three times with kinase assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 1 mM DTT, 0.1% Triton X-100, 10 mM MgCl<sub>2</sub>). Samples were then incubated 15 min at 30°C in 30  $\mu$ l of reaction mix containing 50  $\mu$ M ATP, 25 mM MgCl<sub>2</sub>, 25 mM  $\beta$ -glycerophosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 0.5  $\mu$ g JNK1 in kinase assay buffer, after which 2  $\mu$ g GST-c-Jun and 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP were added, and the reaction was further incubated for 30 min at 30°C. The reaction was terminated by addition of Laemmli sample buffer followed by boiling. Samples were resolved on 12.5% SDS-PAGE and autoradiographed.

## 2.7. JNK phosphatase assay

The assay was done as previously described [3]. Briefly, JNK was activated with various treatments and further phosphorylation of JNK was inhibited by addition of 4  $\mu$ M staurosporine (Sigma). The dephosphorylation kinetics of JNK was detected by Western blotting using a phospho-JNK-specific antibody.

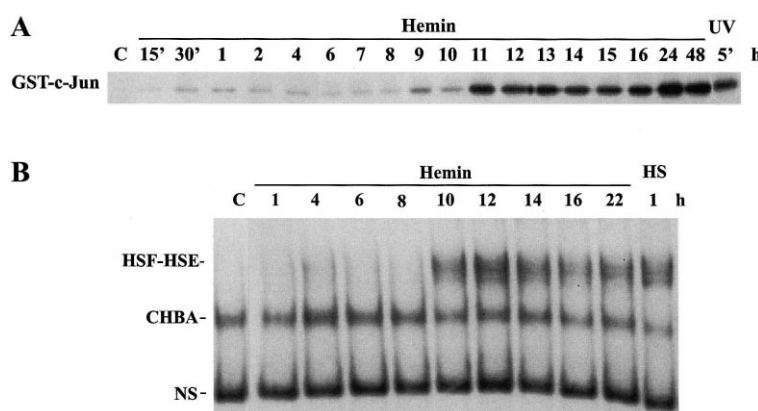


Fig. 1. Activation of JNK follows the HSF2 DNA binding activity during hemin-induced erythroid differentiation of K562 cells. A: An in vitro immunocomplex kinase assay for JNK was performed with extracts from untreated (C) K562 cells, and from K562 cells treated for the indicated time periods with hemin (40  $\mu$ M) or with UV irradiation. B: Whole cell extracts (12  $\mu$ g) from control (C) K562 cells, and from K562 cells treated for the indicated time periods with hemin (40  $\mu$ M) or heat shock (HS; 42°C) were analyzed by gel mobility shift assay. HSF-HSE indicates the specific inducible HSF–HSE complex, CHBA indicates the constitutive HSE binding activity, NS denotes the non-specific protein–DNA interactions.

### 3. Results and discussion

To study JNK activities during erythroid differentiation of K562 cells, hemin-treated cells were analyzed by an *in vitro* JNK activity assay using bacterially expressed GST-c-Jun as a substrate (Fig. 1A). The JNK activity increased markedly after 11 h of hemin treatment ( $\sim 7$ -fold), and by 24 h, the JNK activity was  $>15$ -fold compared to the control levels. Immunoblot analysis of total JNK protein levels showed no changes during hemin treatment (data not shown). The kinetics of HSF2 DNA binding activity was determined using gel mobility shift assay. As shown in Fig. 1B, after 10 h treatment with hemin a prominent and sustained HSF2 DNA binding activity was evident, which is in agreement with our earlier results [10,15,20]. The activation kinetics varied between separate experiments, but in all experiments HSF2 DNA binding activity was observed 1–2 h before the JNK activation.

The concomitant activation of HSF2 and JNK prompted us to examine hemin-induced JNK activity in 2 $\alpha$ -C7 and 2 $\beta$ -D5 cell lines stably overexpressing HSF2- $\alpha$  and HSF2- $\beta$ , respectively (Fig. 2A), since in previous studies we have shown that the hemin-induced HSF2 activation is repressed in HSF2- $\beta$ -overexpressing cells (2 $\beta$ -D5) [20]. As compared to the parental K562 cells, the hemin-induced activation of both HSF2 (Fig. 2B, left panel) and JNK was completely abolished in 2 $\beta$ -D5 cells, whereas UV irradiation led to a clear induction of JNK activity (Fig. 2C). In contrast to the 2 $\beta$ -D5 cells, in the HSF2- $\alpha$ -overexpressing cell line 2 $\alpha$ -C7, which shows a normal activation of HSF2 (Fig. 2B, middle panel) [20], JNK was equally well activated by hemin as in the parental K562 cells (Fig. 2D). To demonstrate that the absence of hemin-induced JNK activity in 2 $\beta$ -D5 was not the result of decreased JNK levels, Western blot analysis for JNK was carried out in parallel (Fig. 2E). This confirms that specific JNK activity is not elevated in 2 $\beta$ -D5 cells. We also tested whether the inhibitory effect of HSF2- $\beta$  could be affected by overexpression of HSF2- $\alpha$ . However, the hemin-induced JNK activation could not be restored by increasing the amount of HSF2- $\alpha$  in 2 $\beta$ -D5 cells (Fig. 2F).

To study the upstream regulatory pathways leading to JNK activation upon hemin-mediated erythroid differentiation of

K562 cells, we analyzed the activity of transfected upstream JNK kinases, MKK4 and MKK7, by employing *in vitro* two-step immunocomplex kinase assays. As shown in Fig. 3A, MKK4 was strongly ( $>5$ -fold) activated by hemin with similar kinetics as JNK, whereas mock transfectants displayed no kinase activity. In contrast to MKK4, MKK7 was not activated during hemin treatment, whereas hyperosmotic shock with 0.5 M NaCl induced a strong activation of MKK7

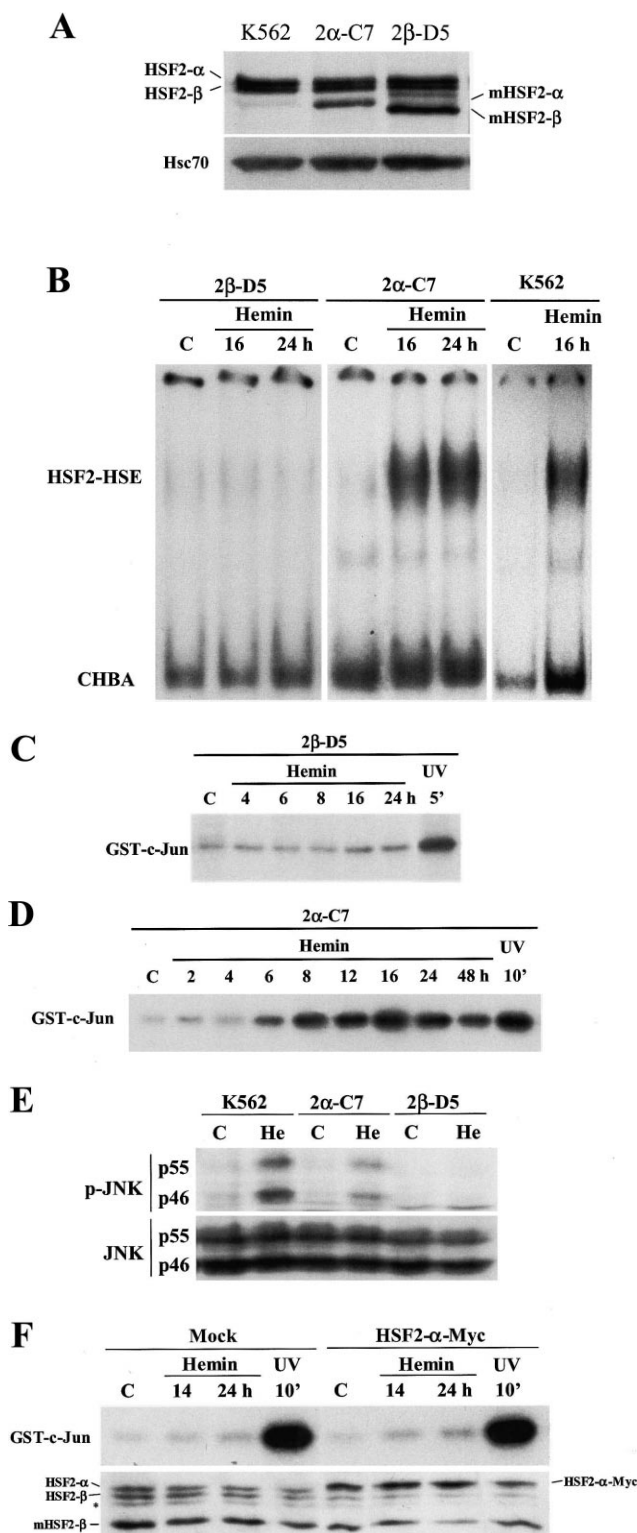


Fig. 2. Inhibition of JNK activation in K562 cells overexpressing the HSF2- $\beta$  isoform. A: Western blot analysis of HSF2 was performed from lysates of K562, 2 $\alpha$ -C7 and 2 $\beta$ -D5 cells. Hsc70 was blotted to demonstrate equal loading. HSF2- $\alpha$ / $\beta$ : endogenous HSF2 isoforms; mHSF2- $\alpha$ / $\beta$ : stably transfected mouse HSF2- $\alpha$ / $\beta$ . B: Gel mobility shift assays were performed as described in Fig. 1 for cell lysates from 2 $\beta$ -D5 (left panel) and 2 $\alpha$ -C7 (right panel) cells. C: An *in vitro* immunocomplex kinase assay for JNK was performed from untreated (C), hemin-treated (40  $\mu$ M) or UV-treated K562 cells stably overexpressing the mouse HSF2- $\beta$  isoform (2 $\beta$ -D5). D: Analysis of JNK activities in control (C) and hemin-treated (40  $\mu$ M) K562 cells overexpressing the HSF2- $\alpha$  isoform (2 $\alpha$ -C7) was performed as in B. E: Western blot analysis of phosphorylated JNK was performed from lysates of untreated (C) or hemin-treated (He; 40  $\mu$ M, 16 h) K562, 2 $\alpha$ -C7 and 2 $\beta$ -D5 cells. Total JNK levels are shown by JNK immunoblot. F: 2 $\beta$ -D5 cells were mock-transfected or transfected with HSF2- $\alpha$ -Myc and treated as indicated. Analysis of JNK activities was done as in C. Western blot analysis of HSF2. HSF2- $\alpha$ / $\beta$ : endogenous HSF2 isoforms; mHSF2- $\beta$ : stably transfected mouse HSF2- $\beta$ ; HSF2- $\alpha$ -Myc: transiently transfected human HSF2- $\alpha$ -Myc; \*: unspecific band or a proteolytic product.

(Fig. 3B). It has been shown by Meriin and co-workers [3] that certain protein-damaging stresses can activate JNK via another regulatory mechanism, namely phosphatase inhibition. To study the rate of JNK dephosphorylation upon hemin treatment, staurosporine, a non-specific kinase inhibitor, was used to block further phosphorylation of JNK. Western blotting with phospho-JNK-specific antibody revealed a fast JNK dephosphorylation in hemin-treated K562 cells, which was comparable to that detected during UV-induced JNK activation (Fig. 3C, upper panel). Already 5 min treatment with staurosporine resulted in almost total dephosphorylation of JNK in cells exposed to either hemin or UV, whereas in cells exposed to heat shock, JNK remained strongly phosphorylated even after 20 min of staurosporine. The same result was obtained when the rate of JNK dephosphorylation was detected by *in vitro* immunocomplex kinase assay (data not shown).

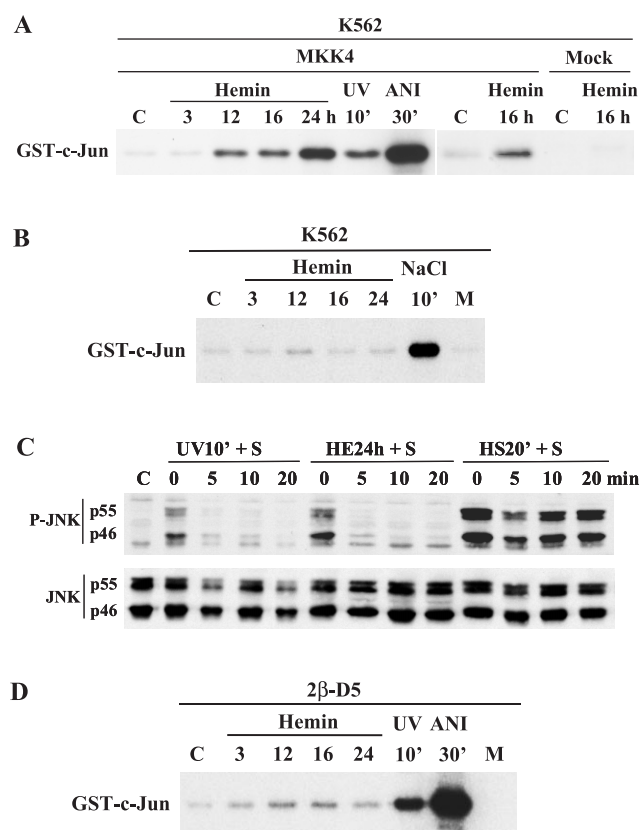


Fig. 3. Hemin-induced JNK activation is mediated via MKK4 and overexpression of HSF2- $\beta$  blocks the activation of MKK4. A: A two-step *in vitro* immunocomplex kinase assay for MKK4 was performed with extracts of untreated K562 cells (C), K562 cells treated with hemin (40  $\mu$ M), UV (10') or anisomycin (ANI; 5  $\mu$ g/ml) 40 h after transfection of a plasmid expressing GST-tagged wild-type MKK4 or mock transfection. B: A two-step *in vitro* immunocomplex kinase assay for MKK7 was performed as in A after transfection of a FLAG-tagged wild-type MKK7. Osmotic shock (NaCl, 0.5 M, 10 min) was used as a positive control. M indicates mock-transfected cells. C: Western blot analysis of the phosphorylation states of JNK. For the analysis of the JNK dephosphorylation kinetics, JNK was activated with UV (10 min), hemin (40  $\mu$ M, 24 h), and heat shock (HS; 45°C, 20 min), and further phosphorylation was blocked with staurosporine (S; 4  $\mu$ M). Western blot analysis of the total JNK levels is shown in the lower panel. D: An identical procedure to that in A was performed in 2 $\beta$ -D5 cells stably overexpressing HSF2- $\beta$ .

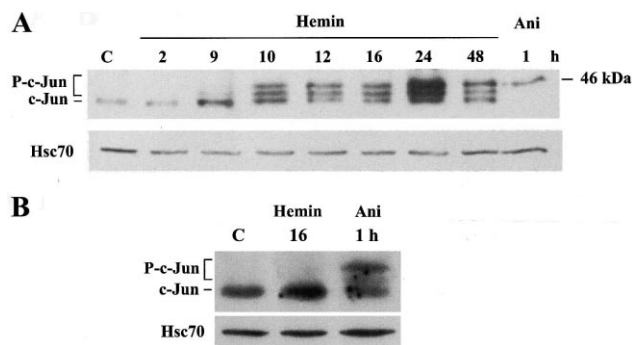


Fig. 4. c-Jun is a putative target for hemin-induced JNK activation. A: Western blot analysis showing the expression levels and phosphorylation states of c-Jun. Cell lysates from untreated K562 cells (C), and K562 cells treated with hemin (40  $\mu$ M) or anisomycin (ANI; 5.0  $\mu$ g/ml) were subjected to SDS-PAGE and immunoblot analysis using an antibody detecting the total c-Jun protein. The different phosphorylation states of c-Jun can be seen as more slowly migrating bands on the gel (P-c-Jun). Hsc70 protein levels from the same samples are shown in the lower panel as a loading control. B: Western blot analysis showing the expression levels and phosphorylation states of c-Jun in cell lysates of untreated (C), hemin-treated (He; 40  $\mu$ M) and anisomycin-treated (ANI; 5.0  $\mu$ g/ml) 2 $\beta$ -D5 cells. Hsc70 is shown as a loading control.

To study whether the HSF2- $\beta$ -mediated suppression of JNK activation acts directly on JNK or further upstream, the activity of MKK4 was determined in 2 $\beta$ -D5 cells. As shown in Fig. 3D, no hemin-induced activation could be observed in these cells, although the cells displayed a marked activation of MKK4 upon stimulation with anisomycin or UV. These results show that the signaling pathway involving MKK4 was affected by overexpression of the inhibitory HSF2- $\beta$  isoform.

To test whether hemin-induced JNK activation played a role in the transcriptional regulation of gene expression during erythroid differentiation of K562 cells, we analyzed the expression and phosphorylation states of c-Jun by immunoblotting with an antibody detecting total c-Jun levels. The different phosphorylation states of c-Jun protein could be seen as several more slowly migrating bands. By 10 h of hemin treatment, the phosphorylated forms of c-Jun could be readily detected and their intensities were further increased by 24 h of hemin treatment (Fig. 4A, upper panel). The amounts of Hsc70, a constitutively expressed member of the 70 kDa Hsp family, are shown as a control for equal loading of samples (Fig. 4A, lower panel). The phosphorylation of c-Jun was repressed in HSF2- $\beta$  overexpressing K562 cells, consistent with the absence of JNK activation (Fig. 4B).

The present study shows a significant activation of the JNK pathway during the erythroid differentiation process of K562 erythroleukemia cells. Previously hematopoietic cytokines, such as erythropoietin (Epo), regulating erythroid differentiation have been shown to activate JNKs in Epo-dependent mouse cell lines [23,24]. In these experiments, the mode of JNK activation appears to be somewhat different from the activation observed by us, because the activation induced by Epo was rapid and transient, which indicates that different mechanisms may be employed by distinct modes of differentiation. Also the upstream regulation appears to be different, since Epo-induced JNK activation is not mediated by MKK4 [23].

Activation of JNK is usually a fast and direct signaling



response to stimuli associated with cellular stresses or exposure to pro-inflammatory cytokines [25,26]. In comparison to stress- or cytokine-mediated activation, the hemin-induced JNK activation is a very slow process with sufficiently delayed kinetics to allow for synthesis of new proteins, whose expression could be regulated by HSF2. Although we cannot dissect whether the inhibitory effect of HSF2- $\beta$  on JNK signaling is mediated directly by preventing HSF2 activity, or indirectly by suppressing erythroid differentiation, our study suggests that the activation of HSF2 could be a crucial step leading to JNK activation during this differentiation process.

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